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MANAGER EXAMINATION SUPPORT AND SALES

Regulation 3.2

The Walter and Eliza Hall Institute of Medical Research

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A method of treatment and an animal model useful for same"

The invention is described in the following statement:

A METHOD OF TREATMENT AND AN ANIMAL MODEL USEFUL FOR SAME

The present invention relates generally to a method of treatment and to an animal model for the identification of molecules and genetic sequences useful in the methods of treatment including inducing or reducing fertility of male animals. More particularly, the present invention contemplates a method for the treatment of infertility or a method of reducing fertility and even more particularly for modulating spermatogenesis in an animal or avian species. There is also provided an animal model comprising a mutation in at least one allele of *bcl-w* or in a gene associated with *bcl-w*. Such animals fail to undergo productive spermatogenesis and can be used to screen for therapeutic molecules including genetic sequences capable of inducing, enhancing or otherwise facilitating spermatogenesis in said animals as well as a model for molecules and genetic sequences which can induce infertility.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Considerable effort has and continues to be expended on therapeutic protocols for the treatment of genetic based disorders. To facilitate the rationale design of such therapeutic protocols, scientists first need to understand and elucidate the biochemical and genetic details of intracellular pathways and physiological processes. Several key regulators have been identified which have involvement in intracellular pathways and physiological processes. A particularly important group of proteins is the Bcl-2 family of proteins.

Bcl-2 is a 26 kDa cytoplasmic protein encoded by the *bcl-2* gene translocated to the IGH locus in human follicular lymphoma and is regarded as the prototypic mediator of cell survival (7). The

Bcl-2 proteins have a role in controlling cellular apoptosis. Apoptosis is a morphologically distinctive and genetically programmed process of cell death (1) and plays an important role in embryogenesis, tissue homeostasis and the immune system.

The mammalian genome contains a range of genes homologous to *bcl-2* but which differ in function. For example, *bcl-x*$_L$ blocks apoptosis (2) whereas *bax* and *bak* inhibit the survival function of *bcl-2* and *bcl-x*$_L$ (3, 4, 5, 6). Due to the potential importance of the cell apoptosis controlling genes in the treatment of cancers and in the treatment of degenerative disorders, there is a profound interest in identifying new genes which are homologous to *bcl-2* in structure and function.

One such recently identified gene is *bcl-w* which is involved in enhancing cell survival (see International Patent Application Number PCT/AU97/00199, filed 27 March, 1997 and incorporated herein by reference).

Like Bcl-2, the Bcl-w protein inhibits apoptosis. Enforced expression of *bcl-w* in lymphoid and myeloid cells markedly enhances their ability to withstand diverse cytotoxic insults.

In work leading up to the present invention, the inventors undertook *bcl-w* gene disruption studies in mice. It has now been surprisingly discovered that mice deficient for *bcl-w* and/or a gene associated with *bcl-w* failed to undergo productive spermatogenesis and are infertile without showing any other major abnormality. The mice provide, therefore, a useful model for studying infertility in animal and avian species.

Accordingly, one aspect of the present invention is directed to a modified animal or avian species exhibiting reduced levels of a Bcl-w protein and/or a protein associated with Bcl-w or a derivative or homologue thereof, wherein said animal or avian species has an incapacity or a reduced capacity to induce or facilitate spermatogenesis.

Reference herein to a "Bcl-w" protein includes reference to a protein having an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or an animo acid sequence

having approximately 47% or greater similarity to either of SEQ ID NO:2 or SEQ ID NO:4. The present invention extends, therefore, to Bcl-w with an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 as well as homologues, analogues and derivatives having at least about 47% similarity to the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4. The Bcl-w protein or its homologues or derivatives are encoded by a nucleotide sequence substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence having at least 47% similarity thereto and/or which is capable of hybridising thereto under low stringency conditions at 42°C. All such derivatives and homologues are encompassed by the terms "Bcl-w" or *"bcl-w"*.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels.

Reference herein to a low stringency at 42°C includes and encompasses from at least about 1% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions.

Preferably, the percentage similarity at the amino acid or nucleotide levels is between 48% and 100% inclusive such as approximately 50% or 55%, 59% or 65%, 70% or 75%, 80% or 85%,

90% or 95% or greater than 96% or a percentage similarity there between.

A gene associated with *bcl-w* or a protein associated with Bcl-w includes the gene which is approximately 9.2 kb down stream of *bcl-w* exon 3 and which has homology to the *Drosophila* rox gene (13). Fusion RNA transcripts have been observed between *bcl-w* and *rox* and, hence, disruption of the rox gene or its transcript or translation production may impact on *bcl-w* expression or Bcl-w activity. The present invention extends, therefore, to targeting Rox, *rox*, *bcl-w-rox* fusion transcripts and Bcl-w-Rox fusion translation products. The present invention extends to other genes associate with *bcl-w* at the regulation, transcription or proximity levels.

Preferably, the Bcl-w protein is of mammalian origin such as from humans, primates, livestock animals (eg. sheep, cows, horses, pigs), companion animals (eg. cats, dogs), laboratory test animals (eg. rabbits, mice, rats, guinea pigs) and captive wild animals (eg. foxes, deer, kangaroos). However, the present invention also extends to non-mammalian homologues of Bcl-w such as from avian species, fish and reptiles. Generally, when producing a modified animal, the effector molecules to reduce Bcl-w activity or expression are identified on the basis of a Bcl-w from the same species. However, an effector molecule against, for example, murine Bcl-w may also be used against human Bcl-w. Both types of effector molecules are contemplated by the present invention and are referred to as heterologous or homologous effector molecules. Similar comments apply with respect to a gene associated with *bcl-w* or a protein associated with Bcl-w.

According to a particularly preferred embodiment, there is provided a modified animal exhibiting reduced levels of Bcl-w or a derivative or homologue thereof and/or of a protein associated with Bcl-w wherein said Bcl-w or its derivative or homologue comprises an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or an amino acid sequence having at least about 47% similarity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 and wherein said modified animal has a incapacity or a reduced capacity to induce or facilitate productive spermatogenesis.

In a related embodiment, there is provided a modified animal exhibiting reduced levels of Bcl-w

or a derivative or homologue thereof and/or of a protein associated with Bcl-w wherein said Bcl-w or its derivative or homologue is encoded by a nucleotide sequence substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence having at least 47% similarity thereto and/or which can hybridise to SEQ ID NO:1 or SEQ ID NO:3 under low stringency conditions at 42°C.

The "modified" animal may be modified at the level of Bcl-w family protein activity or at the genetic level of the *bcl-w* gene. In regards to the former, the present invention contemplates the administration of a range of antagonists to Bcl-w protein activity resulting in reduced or substantially total removal of Bcl-w protein activity. For example, a vaccine may be administered containing Bcl-w protein or an immunogenic derivative thereof to induce antibodies to endogenous Bcl-w protein. Alternatively, a molecule identified from natural product screening capable of acting as an antagonist may be employed. Due to the intracellular nature of Bcl-w, antagonists are generally small molecules or in a form capable of entry into cells. A particularly important potential antagonist is a molecule containing a BH3 amino acid motif. The term "BH" stems from "Bcl-2 Homology" and relates to regions of homology between Bcl-2 proteins (reviewed by Kroemer (8)). The BH3 domain is capable of binding to Bcl-2 and related molecules. Accordingly, a small molecule, for example, comprising a BH3 motif may provide antagonist activity towards Bcl-w. Similar considerations apply in respect of a gene or protein associated with *bcl-w* or Bcl-w, respectively.

According to another aspect of the present invention there is provided a modified animal exhibiting an incapacity or a reduce capacity to induce or facilitate productive spermatogenesis said modification comprising the administration to said animal of an antagonistic effective amount of a molecule capable directly or indirectly of antagonising Bcl-w protein activity or the ability of a derivative or homologue of Bcl-w.

Examples of molecules directly affecting Bcl-w protein activity include an antibody, a soluble receptor for Bcl-w protein and a chemical found from natural product screening. An example of a molecule indirectly affect Bcl-w family protein activity includes a Bcl-w protein or an immunogenic derivative thereof capable of inducing an immune response against an endogenous

Bcl-w protein. Another example is a molecule which targets a gene or protein associated with *bcl-w*/Bcl-w. As stated above, these molecules may need to be modified to permit entry into target cells.

In a related embodiment, there is provided a composition capable of inducing infertility or reducing fertility in an animal, said composition comprising a direct or indirect antagonist of a Bcl-w protein.

Reference to "natural product screening" includes products identified from sources such as but not limited to coral, soil, seabeds and sea water, bacteria, yeasts, plants and river water and river beds.

The composition of this aspect of the present invention may also comprise one or more carriers and/or diluents. Preferably the carriers are pharmaceutically acceptable.

The target animals are as stated above such as humans, primates, livestock animals, laboratory test animals and companion animals. The preferred modified animal, however, for the purposes of an *in vivo* model is a mouse, rat, rabbit, guinea pig, sheep or pig. The most preferred animal is a mouse.

Another aspect of the present invention relates to the genetic reduction in Bcl-w protein levels. According to this aspect of the present invention, there is provided a genetically modified animal comprising a mutation in one or more alleles of a gene encoding a Bcl-w protein and/or of a gene associated with Bcl-w protein.

In a related embodiment, there is provided a genetically modified animal comprising a mutation in one or more alleles of a gene comprising a sequence of nucleotides substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence having at least about 47% similarity thereto and/or a sequence which is capable of hybridising to SEQ ID NO:1 or SEQ ID NO:3 under low stringency conditions at 42°C.

Preferably, in order to observe the infertility phenotype, the animal model comprises an animal with a mutation in both alleles of *bcl-w* and is referred to as *"bcl-w$^{-/-}$"*. An animal with a mutation in one copy of the gene is referred to as "bcl-w$^{+/-}$". A bcl-w$^{+/-}$ is also useful as a carrier for the bcl-w$^{-/-}$ genotype.

In accordance with the present invention, animals and in particular mice carrying a mutation in the *bcl-w* gene have normal populations of lymphoid, myeloid and erythroid cells in bone marrow, spleen, thymus and peripheral blood and normal numbers of haematopoietic progenitors in bone marrow. Adult female bcl-w$^{-/-}$ mice are fertile. However, adult male bcl-w$^{-/-}$ mice are infertile and have small testes. There are no other major abnormalities as determined by, for example, histological examination. The bcl-w$^{-/-}$ mice grow more slowly after puberty than wild-type littermates. The structure of the seminiferous tubules of adult *bcl-w$^{-/-}$* mice is disorganised and the tubules are difficult to categorise according to the normal spermatogenic cycle. Heterogeneous degeneration of all germ cell types is evident, with some degenerating giant cells visible in the tubule lumen. While some round spermatids are present, there are few metamorphosing spermatids and no mature sperm. Seminiferous tubules of *bcl-w$^{-/-}$* mice contain increased numbers of apoptotic nuclei which label with the TUNEL technique, compared to tubules of wild-type littermates. The testes of 2 week old and 4 week old *bcl-w$^{-/-}$* mice appear grossly normal, contain some metamorphosing spermatids and do not contain obvious accumulations of any cell type.

The term "mutation" is used in its broadest sense and includes a single or multiple nucleotide substitution, deletion and/or addition to *bcl-w* or to a region controlling *bcl-w* expression such as a promoter, polyadenylation signal or regulatory gene. The mutation generally results in no active Bcl-w protein being produced or substantially reduced levels of Bcl-w protein being produced. The mutation may also involve a splice variant. The mutation may also be outside the *bcl-w* gene but in a gene associated with *bcl-w* such as the *rox* gene. The term *bcl-w$^{-/-}$* denotes the absence of a functional Bcl-w. For convenience, it is also used to cover reduced levels of Bcl-w such as in the case of the administration of an antagonist of Bcl-w or if antisense molecules are used to induce a transient reduction in Bcl-w levels.

In a particularly preferred embodiment, a substantial portion of the gene has been deleted through, for example, homologous recombination. One particularly useful method is depicted in Figure 4. According to this preferred method a plasmid targeting vector is prepared (eg. denoted *lox-neo bcl-w)* and transfected into embryonic stem (ES) cells. ES cell lines carrying one copy of the targeted *bcl-w* locus are generated and injected into blastocysts to produce chimeric mice. A targeting vector is preferably designed to replace almost the entire *bcl-w* coding sequence with a *pgk-neo* expression cassette. The *pgk-neo* cassette is bounded by sites (*lox*P) that allow its subsequent excision by the action of the bacteriophage Cre recombinase. In order to achieve this, chimeric mice carrying the *bcl-w* mutation have been bred with mice expressing a *Cre* transgene. The correct disruption of the *bcl-w* locus by homologous recombination and removal of the selectable marker by Cre-mediated recombination is confirmed by polymerase chain reaction and Southern blotting. Subsequent breeding generates *bcl-w*$^{-/-}$ mice. A similar approach can be used to mutate a gene associate with *bcl-w.*

There are a number of other mechanisms for generating bcl-w$^{-/-}$ mice or bcl-w$^{+/-}$ mice and all these are encompassed by the present invention.

In addition, the present invention further contemplates transient disruption of the *bcl-w* gene through use of antisense molecules, ribozymes and deoxyribozymes. Viruses may also be employed to introduce antisense molecules or other molecules capable of disrupting function of the *bcl-w* gene. All such genetic molecules are encompassed by the present invention.

Another aspect of the present invention contemplates a method of producing a genetically modified animal substantially incapable of producing Bcl-w, said method comprising introducing a genetic sequence into ES cells, which genetic sequence targets the *bcl-w* gene or a gene associate with *bcl-w* and introducing said ES cells into blastocysts to produce chimeric mice.

The genetic sequence permits excision of the *bcl-w* gene or a selectable marker or specific region within or associated with the *bcl-w* gene by, for example, Cre recombinase.

Preferably, the animal is a mouse.

The ES cells may be from the recipient animal (allergenic) or from a different animal of the same species (heterogenic).

The modified animals of the present invention are particularly useful in screening for genetic or non-genetic molecules capable of restoring fertility. They are also useful as a model for studying the effects of infertility and in the rationale design of molecules capable of inducing infertility.

The bcl-$w^{-/-}$ mutation may also be linked to a "reporter" gene, such as could be used to illustrate expression of *bcl-w* in adult male mice and/or in mouse embryos. For breeding and screening purposes, such a readily identifiable marker would greatly facilitate the identification of bcl-$w^{-/-}$ mice.

A further embodiment of the present invention contemplates transgenic animals such as mice containing a genetic sequence operably linked to a testis-specific promoter, which genetic sequence is capable of disrupting the *bcl-w* gene or *bcl-w* gene expression or expression of a gene associated with *bcl-w* in the testis.

Yet a further embodiment of the present invention is directed to a modified animal comprising a mutation in a gene corresponding to *bcl-w* or a derivative or homologue thereof or in a gene associated with *bcl-w* wherein an adult male of said animal exhibits the following characteristics:

(i) is substantially infertile;
(ii) possesses disorganised seminiferous tubules;
(iii) exhibits heterogenous degeneration of germ cell types; and
(iv) possesses no other major abnormalities as determined by histological examination.

The *bcl-w* mutation is preferably on chromosome 14q11.

Yet a further embodiment of the present invention contemplates an animal model for studying other degenerative disorders such as but not limited to neurodegenerative disorders. For example, animals such as mice which are bcl-$w^{+/-}$ or *bcl-*$w^{-/-}$ in glial cells may ultimately develop

a neurodegenerative disorder. Such animal models would be useful in screening for genetic and therapeutic molecules capable of treating such degenerative disorders.

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a photographic representation showing paraffin-embedded sections of adult mouse testes stained with haematoxylin and eosin. The top left panel depicts a seminiferous tubule from a wild-type mouse. All germ cell types can be seen in this tubule including spermatogonia, spermatocytes and metamorphosing spermatids. The remaining 3 panels depict seminiferous tubules from *bcl-w*$^{-/-}$ mice. A range of abnormalities are visible in these disorganised tubules, including atypical spermatocytes, low numbers of metamorphosing spermatids and degenerating giant cells.

Figure 2 is a photographic representation showing apoptotic cells within paraffin-embedded sections of adult testes detected by the terminal deoxynucleotidal transferase-mediate dUTP nick end-labelling (TUNEL) technique as previously described (9) and the slides counterstained with haematoxylin. The left panel depicts seminiferous tubules from a wild-type mouse. A single TUNEL +ve (brown staining) spermatogonial nucleus is visible. The right panel depicts seminiferous tubules from a *bcl-w*$^{-/-}$ mouse. Several TUNEL +ve nucleic are visible within these disorganised tubules.

Figure 3 is a photo graphical and diagrammatic representation showing confirmation of the genotype of *bcl-w*$^{-/-}$ mice. The polymerase chain reaction was used to amplify products from the genomic DNA of *bcl-w*$^{+/+}$ mice, *bcl-w*$^{+/-}$ mice and *bcl-w*$^{-/-}$ mice, which were separated by agarose gel electrophoresis. (a) A sense primer complimentary to *bcl-w* sequences within the 5′ homology arm was used with antisense primer complimentary to *bcl-w* sequences within the 3′ homology arm to amplify a 1050 bp product from *bcl-w*$^{+/+}$ and *bcl-w*$^{+/-}$ mice. No product could be amplified from *bcl-w*$^{-/-}$ mice or from a water control. (b) A sense primer complimentary to *bcl-w* sequences within the 5′ homology arm was used with an antisense primer complimentary

to *pGK* promoter sequences within the inserted neomycin resistance cassette to amplify a 1000 bp product from *bcl-w*$^{+/-}$ and *bcl-w*$^{-/-}$ mice. No product could be amplified from *bcl-w*$^{+/+}$ mice or from a water control. (c) A sense primer complimentary to *Neo*R sequences within the neomycin resistance cassette was used with an antisense primer complimentary to *bcl-w* sequences within the 3′ homology arm to amplify a 650 bp product from *bcl-w*$^{+/-}$ and *bcl-w*$^{-/-}$ mice. No product could be amplified from *bcl-w*$^{+/+}$ mice or from a water control. (d) A sense primer complimentary to *bcl-w* sequences within the 5′ homology arm was used with an antisense primer complimentary to *bcl-w* sequences within the region deleted following homologous recombination to amplify a 750 bp product from *bcl-w*$^{+/+}$ and *bcl-w*$^{+/-}$ mice. No product could be amplified from *bcl-w*$^{-/-}$ mice or from a water control.

Figure 4 is a diagrammatic representation depicting the strategy for disrupting the mouse *bcl-w* gene. (a) The top panel depicts the *lox-neo bcl-w* targeting vector. The 5′ and 3′ arms of homology are depicted blue, the thymidine kinase negative selectable marker cassette is depicted green and the neomycin resistance positive selectable marker cassette is depicted yellow. (b) The second panel depicts the wild type mouse *bcl-w* locus. Crossed lines represent homologues recombination between the wild type locus and the targeting vector. Boxes represent the exons of the *bcl-w* gene. Red boxes represent the coding region of the bcl-w gene. (c) The third panel depicts the mutant *bcl-w* gene after homologous recombination. (d) The fourth panel represents the mutant *bcl-w* gene after Cre-mediated recombination has excised the neomycin resistance positive selectable marker cassette.

EXAMPLE 1
GENERATION OF *bcl-w* KNOCKOUT MICE

The *bcl-w* gene is inactivated by homologous recombination. The strategy employed is shown in Figure 4. To promote homologous recombination, a targeting vector denoted *lox-neo bcl-w* is prepared for transfection into embryonic stem (ES) cells. In this vector, two segments of the *bcl-w* locus flank a positive selectable marker, the neomycin resistance gene, *neoR,* which renders transfected ES cells resistant to the antibiotic G418. The vector carries a negative selectable marker, the vaccinia virus thymidine kinase (*tk)* gene, placed outside the *bcl-w* sequences. On non-targeted integration, *tk* sequences are likely to remain with the targeting vector, rendering ES cells sensitive to the antibiotic gancyclovir. This "positive-negative" selection strategy (10) is used to enrich for homologous recombinants at selected loci.

The *lox-neo bcl-w* targeting vector is designed to remove essentially all *bcl-w* sequences and to leave minimal foreign sequences in the final targeted locus. Following homologous recombination, almost the entire *bcl-w* coding region, starting precisely from the initiating ATG, is replaced by a *pgk-neo* expression cassette, bounded by sites (*lox*P) that allow its subsequent excision. Once ES cell clones bearing homologous recombinants have been identified, the *pgk-neo* cassette can be readily excised by exploiting the site-specific recombinase activity of the bacteriophage Cre protein, which deletes all sequences between two tandem *lox*P sites.

The *lox-neo bcl-w* targeting vector is introduced by electroporation into the ES cell line W9.5 (11). Following electroporation, the ES cells are cultured on fibroblasts in a medium containing leukemia inhibitory factor (LIF) to maintain an undifferentiated state, G418 for positive selection and gancyclovir for negative selection. Ten to fourteen days later, about 500 clones were picked and tested for homologous recombination by the polymerase chain reaction and Southern blotting. Of the picked clones, 8 were shown to contain a single correctly targeted insert and to lack extraneous random integrants. Two of these were microinjected into mouse blastocysts, which were introduced into pseudo-pregnant mice. All chimeric progeny having a high ES cell contribution to coat colour were bred and achieved germ line transmission. In order to achieve excision of the *pgk-neo* expression cassette, chimeric mice carrying the *bcl-w* mutation were bred

with mice expressing a *Cre* transgene. The correct removal of the selectable marker by Cre-mediated recombination was confirmed by polymerase chain reaction and Southern blotting. Two breeding lines derived from distinct original ES clones were established, to preclude confounding effects due to extraneous mutations. Subsequent breeding has generated *bcl-w*$^{-/-}$ mice.

EXAMPLE 2
PHENOTYPE OF *bcl-w*$^{-/-}$ MICE

Litters of *bcl-w*$^{-/-}$ mice were born at approximately Mendelian frequencies and were grossly normal in appearance and behaviour. Detailed histological study of these mice revealed no abnormalities in most tissues. The *bcl-w*$^{-/-}$ mice have normal populations of lymphoid, myeloid and erythroid cells in bone marrow, spleen, thymus and peripheral blood and normal numbers of haematopoietic progenitors in bone marrow. Adult female *bcl-w*$^{-/-}$ mice were fertile.

However, adult male *bcl-w*$^{-/-}$ mice were infertile with small testes and grew more slowly after puberty than wild-type littermates. The structure of seminiferous tubules of adult *bcl-w*$^{-/-}$ mice was disorganised and the tubules were difficult to categorise according to the normal spermatogenic cycle. Heterogeneous degeneration of all germ cell types was evident, with some degenerating giant cells visible in the tubule lumen. While some round spermatids were present, there were few metamorphosing spermatids, and no mature sperm (Figure 1). Seminiferous tubules of *bcl-w*$^{-/-}$ mice contained increased numbers of apoptotic nuclei which label with the TUNEL technique, compared to tubules of wild-type littermates (Figure 2). The testes of 2 week old and 4 week old *bcl-w*$^{-/-}$ mice (which are undergoing the first round of spermatogenesis) appeared grossly normal, contain some metamorphosing spermatids and did not contain obvious accumulations of any cell type.

Confirmation of the *bcl-w*$^{-/-}$ genotype is shown in Figure 3.

EXAMPLE 3
EXPRESSION OF PATTERN OF *bcl-w*

In order to determine the expression pattern of *bcl-w* throughout the mouse, homologous recombination experiments are conducted to replace the coding region of *bcl-w* with a readily detectable reporter gene, *LacZ*. The targeting vector, *LacZ bcl-w*, is designed to replace most of the major *bcl-w* coding exon, starting precisely from the initiating ATG, with the *lacZ* gene (encoding *E. coli* β-galactosidase). Because the *LacZ* reporter gene is driven by the *bcl-w* promoter, expression of β-galactosidase and heterozygous mice bearing this construct will closely mimic that of the endogenous bcl-w protein. Sensitive assays are then exploited for β-galactosidase activity to reveal the bcl-w expression pattern.

Sensitive and complementary assays for β-galactosidase activity include the following: (a) the X-gal histochemical assay, which is well suited for whole-mount studies on young embryos and for staining sections of organs from foetal or adult animals; and (b) the flow cytometric (FACS-gal) assay which is a cell-by-cell assay, particularly valuable for analysis of haemopoietic expression, because it can be combined with analysis of cell surface antigens in multi-variant FACS analysis (12). By applying both these methods, a complete picture of *bcl-w* expression throughout the adult and embryonic mouse is obtainable.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH

(ii) TITLE OF INVENTION: A METHOD OF TREATMENT AND AN ANIMAL USEFUL FOR SAME

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 583 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..583

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTT GTA GGT TAT AAG CTG AGG CAG AAG GGT TAT GTC TGT GGA GCT GGC        96
Phe Val Gly Tyr Lys Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
            20                  25                  30

CCC GGG GAG GGC CCA GCA GCT GAC CCG CTG CAC CAA GCC ATG CGG GCA       144
Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
        35                  40                  45

GCT GGA GAT GAG TTC GAG ACC CGC TTC CGG CGC ACC TTC TCT GAT CTG       192
Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
    50                  55                  60

GCG GCT CAG CTG CAT GTG ACC CCA GGC TCA GCC CAG CAA CGC TTC ACC       240
Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
65                  70                  75                  80

CAG GTC TCC GAC GAA CTT TTT CAA GGG GGC CCC AAC TGG GGC CGC CTT       288
Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
                85                  90                  95

GTA GCC TTC TTT CTC TTT GGG GCT GCA CTG TGT GCT GAG AGT GTC AAC       336
Val Ala Phe Phe Leu Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
            100                 105                 110

AAG GAG ATG GAA CCA CTG GTG GGA CAA GTG CAG GAG TGG ATG GTG GCC       384
Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Glu Trp Met Val Ala
        115                 120                 125

TAC CTG GAG ACG CGG CTG GTC GAC TGG ATC CAC AGC AGT GGG GGC TGG       432
Tyr Leu Glu Thr Arg Leu Val Asp Trp Ile His Ser Ser Gly Gly Trp
    130                 135                 140

GCG GAG TTC ACA GCT CTA TAC GGG GAC GGG GCC CTG GAG GAG GCG CGG       480
Ala Glu Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Glu Ala Arg
145                 150                 155                 160

CGT CTG CGG GAG GGG AAC TGG GCA TCA GTG AGG ACA GTG CTG ACG GGG       528
Arg Leu Arg Glu Gly Asn Trp Ala Ser Val Arg Thr Val Leu Thr Gly
                165                 170                 175

GCC GTG GCA CTG GGG GCC CTG GTA ACT GTA GGG GCC TTT TTT GCT AGC       576
Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
```

```
            180                 185                 190

AAG TGA A                                                       583
Lys  *
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 194 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ala Thr Pro Ala Ser Ala Pro Asp Thr Arg Ala Leu Val Ala Asp
  1               5                  10                  15

Phe Val Gly Tyr Lys Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
             20                  25                  30

Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
         35                  40                  45

Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
     50                  55                  60

Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
 65                  70                  75                  80

Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
                 85                  90                  95

Val Ala Phe Phe Leu Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
            100                 105                 110

Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Glu Trp Met Val Ala
        115                 120                 125

Tyr Leu Glu Thr Arg Leu Val Asp Trp Ile His Ser Ser Gly Gly Trp
    130                 135                 140

Ala Glu Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Glu Ala Arg
145                 150                 155                 160

Arg Leu Arg Glu Gly Asn Trp Ala Ser Val Arg Thr Val Leu Thr Gly
                165                 170                 175

Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
            180                 185                 190

Lys  *
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..582

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG CCG ACC CCA GCC TCA ACC CCA GAC ACA CGC GCT CTA GTG GCT GAC          48
Met Pro Thr Pro Ala Ser Thr Pro Asp Thr Arg Ala Leu Val Ala Asp
 1               5                  10                  15

TTT GTA GGC TAT AGG CTG AGG CAG AAG GGT TAT GTC TGT GGA GCT GGG          96
Phe Val Gly Tyr Arg Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
            20                  25                  30

CCT GGG GAA GGC CCA GCC GCC GAC CCG CTG CAC CAA GCC ATG CGG GCT         144
Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
        35                  40                  45

GCT GGA GAC GAG TTT GAG ACC CGT TTC CGC CGC ACC TTC TCT GAC CTG         192
Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
    50                  55                  60

GCC GCT CAG CTA CAC GTG ACC CCA GGC TCA GCC CAG CAA CGC TTC ACC         240
Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
65                  70                  75                  80

CAG GTT TCC GAC GAA CTT TTC CAA GGG GGC CCT AAC TGG GGC CGT CTT         288
Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
                85                  90                  95

GTG GCA TTC TTT GTC TTT GGG GCT GCC CTG TGT GCT GAG AGT GTC AAC         336
Val Ala Phe Phe Val Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
            100                 105                 110

AAA GAA ATG GAG CCT TTG GTG GGA CAA GTC CAG GAT TGG ATC GTG GCC         384
Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Asp Trp Ile Val Ala
        115                 120                 125

TAC CTG GAG ACA CGT CTG GCT GAC TGG ATC CAC AGC AGT GGC GGC TGG         432
Tyr Leu Glu Thr Arg Leu Ala Asp Trp Ile His Ser Ser Gly Gly Trp
    130                 135                 140

GCG GAC TTC ACA GCT CTA TAC GGG GAC GGG GCC CTG GAG GAC GCA CGG         480
Ala Asp Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Asp Ala Arg
145                 150                 155                 160

CGT CTG CGG GAG GGC AAC TGG GCA TGA GTG AGC ACA GTG GTG ACG GGG         528
Arg Leu Arg Glu Gly Asn Trp Ala  *  Val Ser Thr Val Val Thr Gly
                165                 170                 175

GCC GTG GCA CTG GGG GCC CTG GTA ACT GTA GGG GCC TTT TTT GCT AGC         576
Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
            180                 185                 190

AAG TG                                                                  582
Lys
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 193 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Pro Thr Pro Ala Ser Thr Pro Asp Thr Arg Ala Leu Val Ala Asp
  1               5                  10                  15

Phe Val Gly Tyr Arg Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
            20                  25                  30

Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
        35                  40                  45

Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
    50                  55                  60

Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
65                  70                  75                  80

Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
                85                  90                  95

Val Ala Phe Phe Val Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
            100                 105                 110

Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Asp Trp Ile Val Ala
        115                 120                 125

Tyr Leu Glu Thr Arg Leu Ala Asp Trp Ile His Ser Ser Gly Gly Trp
    130                 135                 140

Ala Asp Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Asp Ala Arg
145                 150                 155                 160

Arg Leu Arg Glu Gly Asn Trp Ala  *  Val Ser Thr Val Val Thr Gly
                165                 170                 175

Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
            180                 185                 190

Lys
```

DATED this 16th day of September, 1997

The Walter and Eliza Hall Institute of Medical Research

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

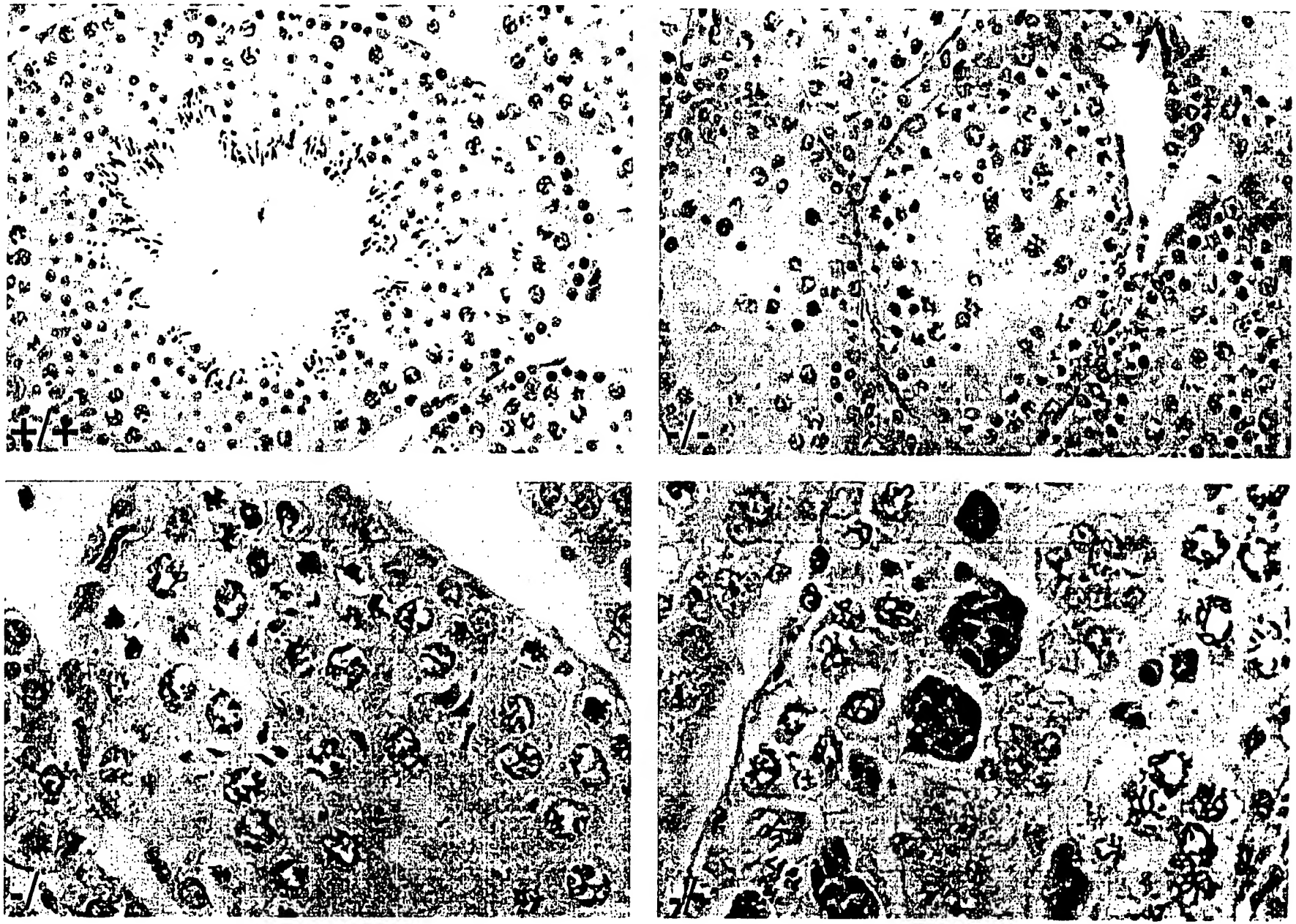
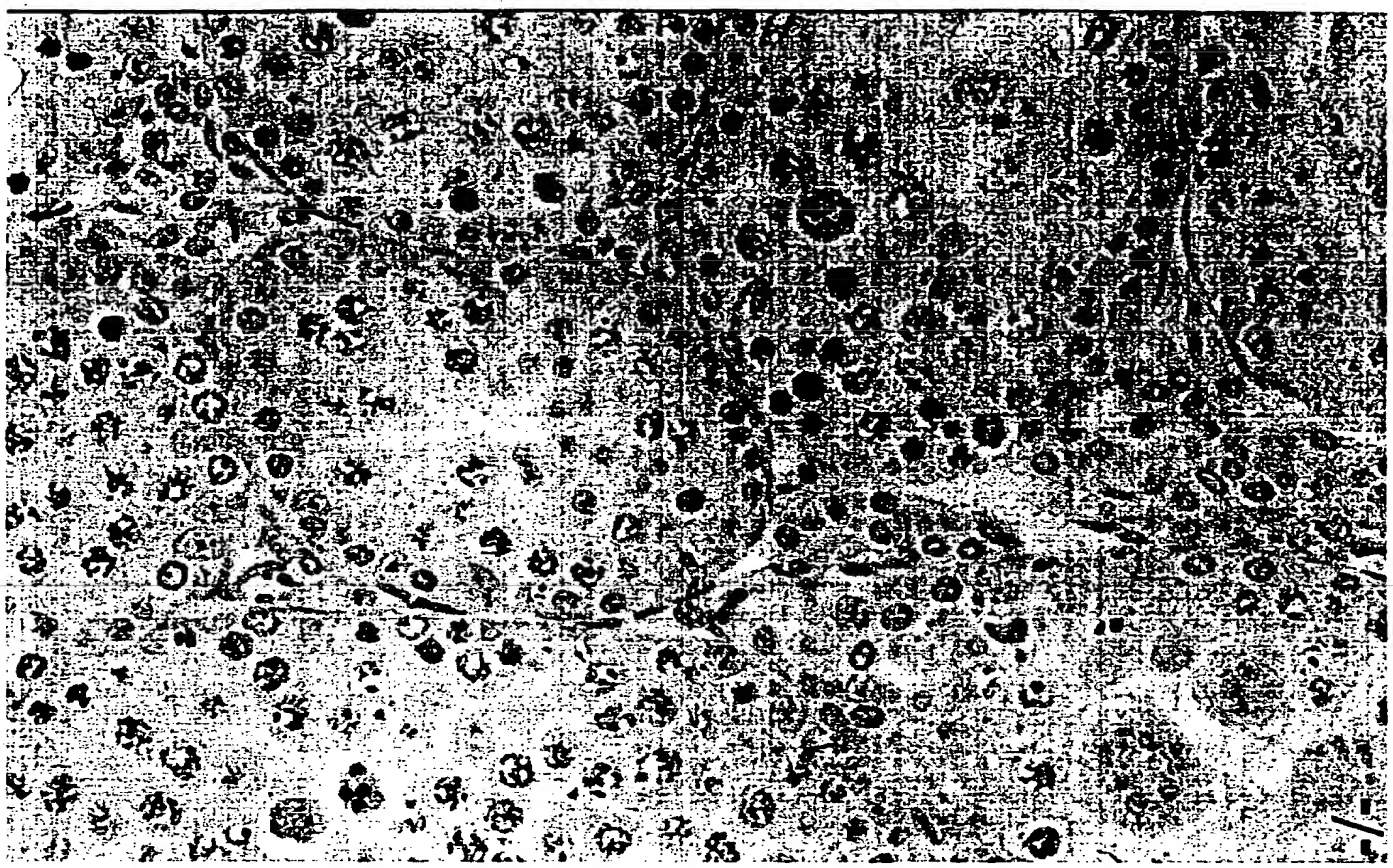


Fig. 1



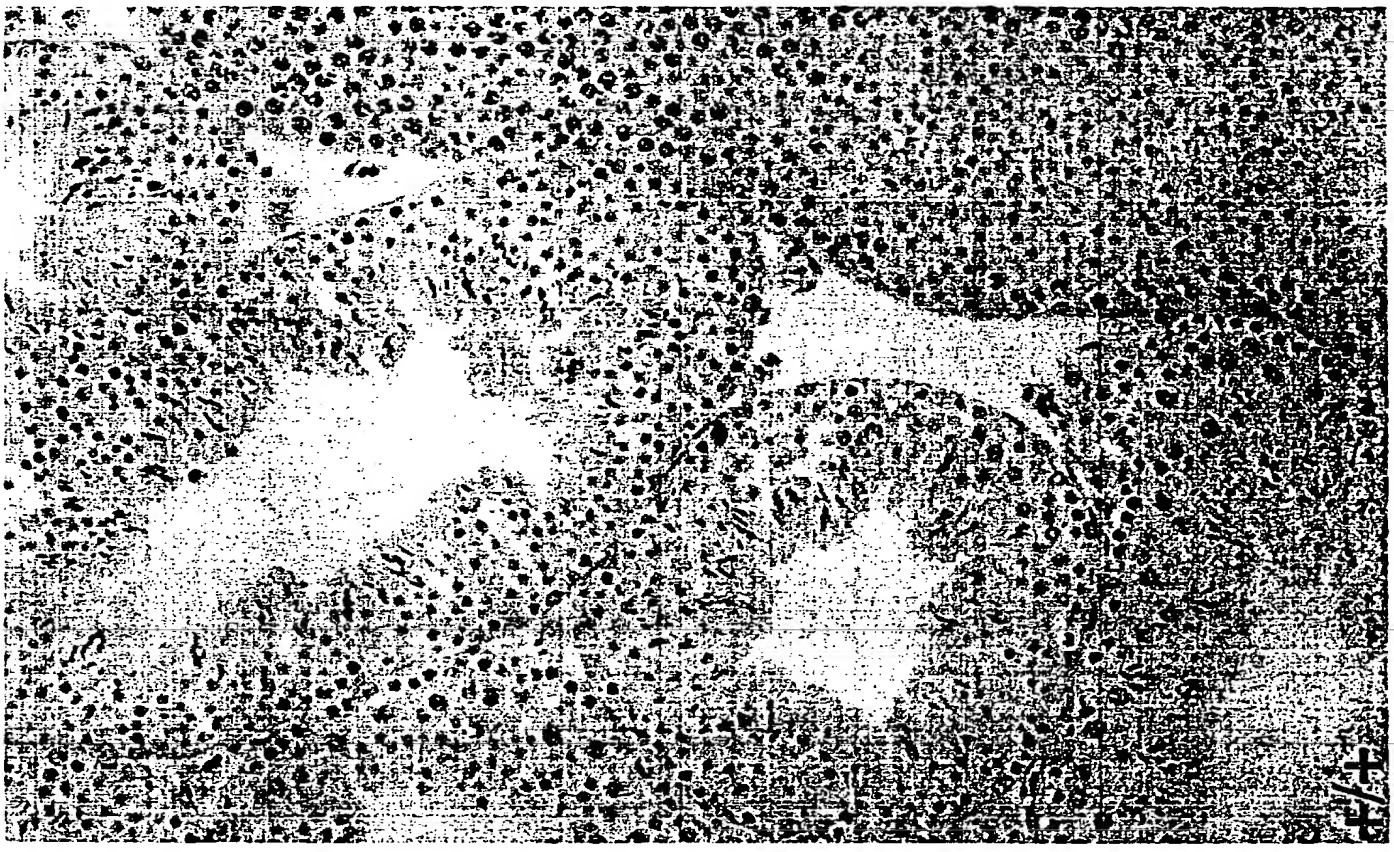
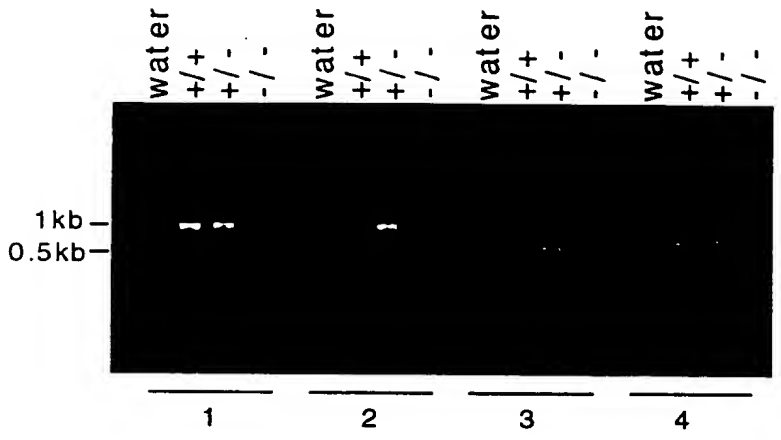
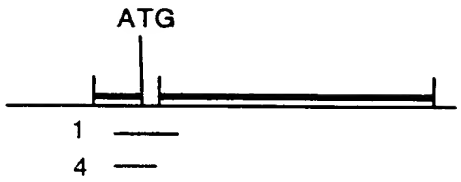


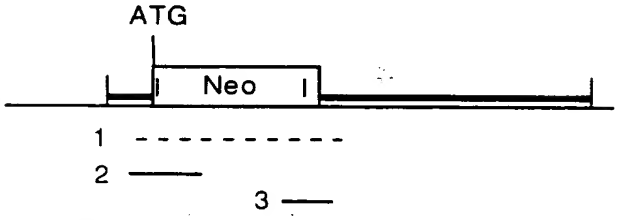
FIG. 2

Fig. 3. Confirmation of the genotype of *bcl-w* $^{-/-}$ mice





Wild-type mouse *bcl-w* gene



Mutant mouse *bcl-w* gene

Fig. 4. Strategy for Disrupting the mouse *bcl-w* gene